Master's Thesis

MicroRNA-21, -126 and -146a as potential novel systemic markers of metabolic syndrome

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PREFACE

This Master's thesis was conducted at The Gerontology Research Center at the Faculty of Sport and Health Sciences, University of Jyväskylä. This thesis is part of the Estrogenic Regulation of Muscle Apoptosis (ERMA) research project.

First, I would like to thank my supervisor Eija Laakkonen for her guidance and support during this Master's thesis. I also want to thank Vuokko Kovanen for accepting me into the ERMA research project and Markku Kauppinen for helping me with the statistical analyses. Also, I would like to thank the people in the laboratory for the helpful assistance.

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Obesity and overweight have reached epidemic proportions. These conditions increase the risk of numerous health problem, including metabolic syndrome (MetS). Hormonal changes during menopause transition may be related to the increased frequency of the MetS. Circulating microRNAs (miRNAs) are easily detectable in blood and have a potential to act as non-invasive biomarkers. MiR-21, miR-126, and miR-146a were selected in this Master's thesis because they are associated with MetS risk factors and menopause-related hormone estradiol (E_2). Hypothesis of this study was that circulating miR-21, miR-126 and miR-146a levels may vary in different MetS risk factor groups and stage of menopause. Participants (n=137) were divided to the MetS risk factor groups; healthy (n=35), risk 1 (n=35), risk 2 (n=33), and MetS (n=34), based on anthropometrics and measured blood characteristics. Menopause groups; pre- (n=45), peri- (n=44) and postmenopause (n=48) were divided based on blood test and menstrual diary. Circulating miRNAs levels were quantified with quantitative polymerase chain reaction (qPCR). The results could not establish distinct association between serum miR-21, miR-126 and miR-146a to the risk factor groups of MetS or to the stage of menopause. However, significant correlation was observed between miR-21 and E_2, miR-21 and glucose and miR-126 and glucose. Based on the results of this thesis, despite the associations found between miRNAs and glucose, the studied miRNAs cannot be considered as markers of MetS.

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Ylipaino lisää riskiä sairastua esimerkiksi sydän- ja verisuonisairauksiin, tukielinsairauksiin sekä metaboliseen oireyhtymään. On selvästi todettu, että naisilla vaihdevuosien hormonaaliset muutokset lisäävät ylipainon riskiä ja tästä syystä myös metabolisen oireyhtymän riskiä. Tässä työssä tutkittiin voidaanko veressä kiertäviä mikroRNA:ita (miRNA) käyttää metabolisen oireyhtymän varhaiseen diagnoosiin. Valitsimme miR-21, miR-126 ja miR-146a tutkimuskohteiksi, koska niiden on osoitettu olevan yhteydessä sekä estrogeenin määrään sekä metabolisen oireyhtymän riskitekijään krooniseen tulehdukseen. Tämän tutkimuksen hypoteesi oli, että miR-21, miR-126 ja miR-146a-tasot voivat vaihdella eri metabolisen oireyhtymän riskitekijäryhmissä ja vaihdevuosien vaiheessa. Osallistujat (n=137) jaettiin antropometristen tulosten ja verikokeiden avulla metabolisen oireyhtymän riskitekijäluokkiin: terveet (n=35), riski 1 (n=35), riski 2 (n=33) ja MetS (n=34). Lisäksi osanottajat jaettiin verikokeiden ja kuukautiskierron perusteella seuraaviin vaihdevuosiryhmiin: pre- (n=45), peri- (n=44) ja postmenopaussi (n=48). Veressä kiertävien miRNA-tasot mitattiin kvantitatiivisella polymeraasiketjureaktiolla (qPCR). Seerumin miR-21, miR-126 ja miR-146a tasoissa ei havaittu tilastollisesti merkitseviä eroja metabolisen oireyhtymän riskitekijäryhmissä eikä vaihdevuosiryhmissä. Tilastollisesti merkitsevä yhteys kuitenkin havaittiin miR-21 ja estradiolin (E_2), miR-21 ja glukoosin sekä miR-126 ja glukoosin välillä. Työn tulosten perusteella tutkittuja mikroRNA:ita ei voida pitää sopivina metabolisen oireyhtymän merkkiaineina.

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ABBREVIATIONS

ANOVA	analysis of variance
BMI	body mass index
cDNA	complementary DNA
CVD	cardiovascular disease
DGCR8	DiGeorge syndrome critical region gene 8
DBP	diastolic blood pressure
E_2	estradiol
FSH	follicle-stimulating hormone
Gluc	glucose
HDL	high density lipoprotein
IDF	International Diabetes Federation
LDL	low density lipoprotein
LH	luteinizing hormone
miR	microRNA
miRNA	microRNA
MetS	metabolic syndrome
RISC	RNA-induced silencing complex
SBP	systolic blood pressure
T2DM	type 2 diabetes mellitus
TC	total cholesterol
TG	triglycerides
qPCR	quantitative real-time polymerase chain reaction

1 INTRODUCTION

Obesity and overweight is a growing global health problem. These conditions increase the risk of numerous health problem, including metabolic syndrome (MetS) and cardiovascular diseases (CVD) (Alberti *et al.* 2009). The frequency of the MetS increases with menopause and may be related to hormonal and metabolic changes that occur during the transition from premenopause to postmenopause. Studies have shown that change in hormonal milieu at menopause is associated with central abdominal fat and enlarged waist circumference (Davis *et al.* 2012, Baber *et al.* 2016). Especially excess visceral adiposity is associated to dyslipidemia, insulin resistance and hypertension (Ouchi *et al.* 2011). Obesity is linked to low-level chronic inflammation which contribute to the development of metabolic disorders. In addition to this, women with MetS are known to be at particularly high risk for CVD (Carr 2003).

This study deals with the potential association of circulating microRNAs (miRNAs) with MetS and menopause. MicroRNAs are found to be associated in variety conditions and diseases (Chen *et al.* 2008). MiRNAs are exceptionally stable in serum/plasma and have potential to serve as new blood-based biomarkers in various conditions (Mitchell *et al.* 2008, Reid *et al.* 2011). In the introduction I will review the hallmarks of MetS, the biology of three miRNAs selected for this study and the hormonal changes during menopause.

1.1 The metabolic syndrome

The MetS is defined as simultaneous presence of several metabolic dysfunctions. The features of MetS include abdominal obesity, insulin resistance, hypertension, dyslipidemia, prothrombotic state and proinflammatory state (Carr 2003, Eckel *et*

al. 2005). The presence of the MetS increases the risk of type 2 diabetes mellitus (T2DM) and CVD (Eckel *et al.* 2005). According to the definition of MetS by an International Diabetes Federation (IDF) a person has MetS when her/his waist circumference (WC) is elevated concurrently occurring with two or more of the four additional MetS risk factors. These additional risk factors are, elevated triglycerides (TG), reduced high density lipoprotein (HDL), raised systolic/diastolic blood pressure (SBP/DBP) and elevated fasting glucose (Gluc) (International Diabetes Federation 2006). There is also a definition of MetS where 3 abnormal findings out of 5 would qualify a person for the MetS (Alberti *et al.* 2009). In this Master's thesis definition of MetS by IDF was used. IDF criteria for clinical diagnosis of the MetS are listed in (Table 1).

Table 1. Diagnostic criteria for the MetS according to IDF. Defined as having the MetS, a person must have elevated WC and any two or more of four additional factors. Table is modified from, The IDF consensus worldwide definition of the metabolic syndrome (International Diabetes Federation 2006).

Risk factor	**Defining level**
Elevated waist circumference	≥ 80 cm (for Europid, women)
Elevated triglycerides (or drug treatment)	≥ 1.7 mmol/l
Redused HDL cholesterol (or drug treatment)	< 1.3 mmol/l (in females)
Elevated fasting glucose (or drug treatment)	≥ 5.6 mmol/l
Elevated blood pressure (or drug treatment)	systolic ≥ 130 and/or diastolic ≥ 85 mmHg

IDF worldwide definition of the metabolic syndrome

Increasing obesity and sedentary lifestyle have risen the MetS prevalence worldwide (Alberti *et al.* 2009). Pathogenesis of the MetS remains unclear but insulin resistance and increased visceral obesity are believed to be related to the underlying pathophysiology of the MetS (Carr 2003, Alberti *et al.* 2009). Patients with metabolic risk factors should be treated with lifestyle intervention such as dietary changes,

weight reduction and increased physical activity, followed by drug treatment if necessary (Eckel *et al.* 2005, Alberti *et al.* 2009).

1.2 MiRNAs

The first small RNAs was discovered by Lee *et al*. (1993). Later these tiny regulatory RNAs were named as microRNAs (Lagos-Quintana *et al.* 2001, Lau *et al.* 2001, Lee and Ambros 2001). MiRNAs are single stranded small non-coding RNAs of ~ 22 nucleotides (nt) in length. They regulate gene expression either through post-transcriptional degradation or translational repression by binding to complementary sites within the 3´-untranslated region (3´-UTR) of target messenger RNA (mRNA) (for review see Ha and Kim 2014). Circulating miRNAs are easily detectable in blood or other body fluids and are stable despite high endogenous RNase activity (Mitchell *et al.* 2008). Majority of blood miRNAs have been suggested to be vesicle-free and bound to Ago2 protein. The remaining miRNAs are exported in extracellular vesicles such as exosomes or apoptotic bodies (Turchinovich *et al.* 2011). Also, it is shown that hormones regulate miRNAs, for example, E_2 (Klinge 2009, Wickramasinghe *et al.* 2009). MiRNAs play important roles in numerous physiologic and pathologic processes such as cancer, vascular and metabolic diseases. In the near future, circulating miRNAs will likely to be used as new biomarkers for detecting, risk estimation or classification for different kinds of diseases (Mitchell *et al.* 2008, Zampetaki and Mayer 2012, Nunez Lopez *et al.* 2016).

1.2.1 Biogenesis of miRNA

As summarised in the review articles Kim *et al.* (2009), Ha and Kim (2014) biogenesis of miRNA proceeds when miRNA genes are transcribed by RNA polymerase II (Pol II) that produce primary transcripts (pri-miRNAs). Pri-miRNAs are regularly several kilobases long and harbour local stem-loop structure where miRNA sequences are embedded. Pri-miRNAs are cropped within the nucleus by Drosha, an RNase III-type nuclease, into ~ 65 nt hairpin-structured precursors (pre-

miRNAs). Drosha requires crucial cofactor DiGeorge syndrome critical region gene 8 (DGCR8) to form a complex named the microprocessor. After Drosha processing, pre-miRNAs are exported into the cytoplasm by Exportin-5 (Exp-5) in complex with Ran-GTP. In cytoplasm pre-miRNAs are cleaved by Dicer, an RNase III-type endonuclease, to generate a duplex miRNA. One strand of the duplex (guide strand) is selected in Argonaute (AGO) loading step and the passenger strand is degraded. Mature miRNA is loaded into a RNA-induced silencing complex (RISC). RISC complex contains AGO proteins and auxiliary proteins such as TAR RNA-binding protein (TRBP) and protein kinase R-activating protein (PACT). RISC complex binds to the 3′-UTR of a target mRNA transcripts. MiRNAs partial complementary to target mRNA leads to translational repression whereas highly complementary leads to mRNA cleavage and degradation. Schematic view of the miRNA biogenesis is presented in (Figure 1).

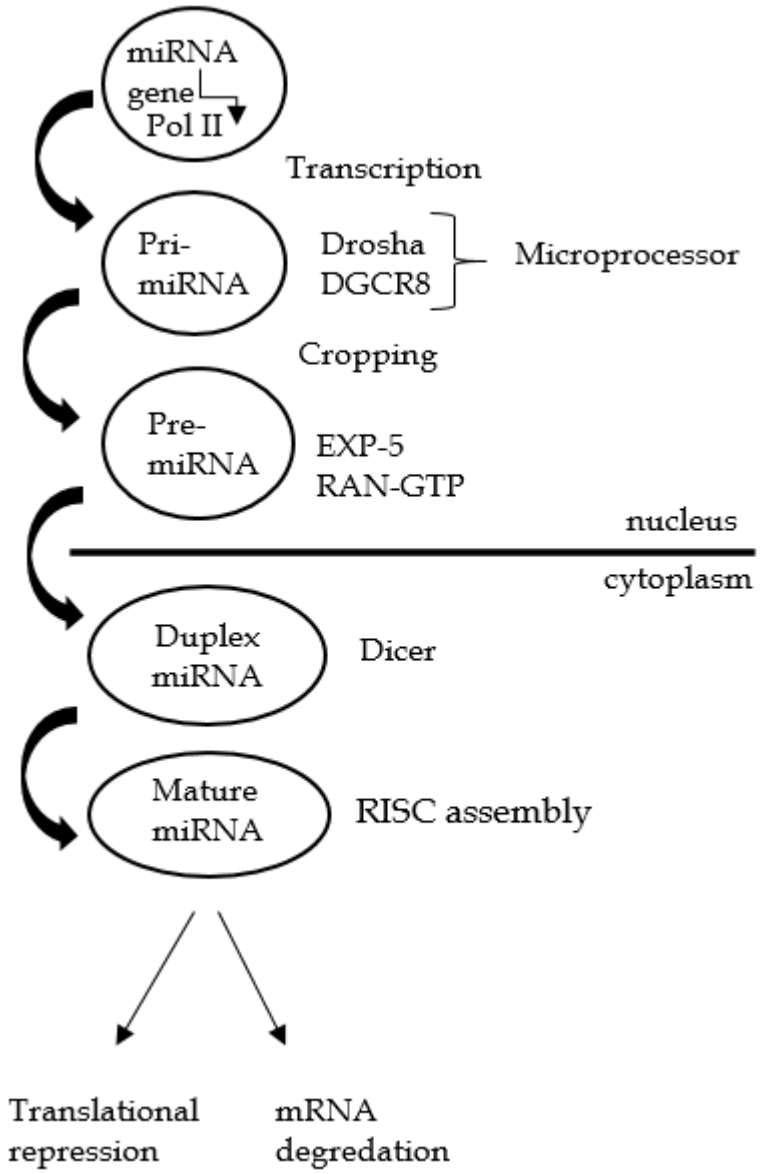

Figure 1. Schematic view of the miRNA biogenesis pathway. MiRNAs are transcribed by RNA polymerase II to produce pri-miRNA. Pri-miRNAs are processed by the Drosha-DGCR8 complex to generate pre-miRNAs. Pre-miRNAs are transported by Exp-5 into the cytoplasm, where they are further spliced into mature miRNA by Dicer. Mature miRNA is loaded into RISCs complex and are directed to a specific target mRNA sequences. The figure is based on the picture from the article, Biogenesis of small RNAs in Animal (for review see Kim *et al.* 2009).

1.2.2 MiR-21

Human miR-21 gene is located on chromosome 17q23.2 and it is known as an oncogenic miRNA due to its overexpression in numerous human malignancies.

Most of its targets are known to be tumor suppressor genes, such as phosphatase and tensin homolog (PTEN), which negatively regulates protein kinase B (Akt) signaling pathway (MacFarlane and Murphy 2010, Musilova and Mraz 2015, Sekar *et al.* 2015). MiR-21 plays important roles in inflammation and endothelial dysfunction which belongs to progression of T2DM and CVD. MiR-21 is elevated in the circulation of T2DM subjects (Olivieri *et al.* 2015, Nunez Lopez *et al.* 2016). Also, Kangas *et al.* (2014) have shown that systemic E_2 concentration affect serum miR-21 levels.

1.2.3 MiR-126

MiR-126 is highly enriched in endothelial cells (ECs). It is involved in angiogenesis and maintenance of vascular integrity, therefore, it is called "angiomiR". Cytogenic location of miR-126 is on chromosome 9q34.3. It is encoded by intron 7 of the epidermal growth factor-like domain 7 (EGFL7) gene (Zampetaki and Mayer 2012, Lopez-Ramirez *et al.* 2016). MiR-126 assists angiogenic growth factors, like vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) by repressing of the sprouty-related protein 1 (Spred-1) and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2/p85-β). These are intracellular inhibitors of the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways (Zampetaki and Mayer 2012). MiR-126 is significantly reduced in the circulation of T2DM patients (Nunez Lopez *et al.* 2016). In addition, it has been demonstrated as a potential marker predicting diabetic vascular and myocardial complications (Villard *et al.* 2015). Also, circulating miR-126 concentration negatively associates with serum E_2 levels (Kangas *et al.* 2017).

1.2.4 MiR-146a

Circulating miR-146a is known as "inflamma-miR" because it is involved in regulating immune and inflammation response. Low-level chronic inflammation has been linked to normal aging and in many age-related diseases, such as CVD, T2DM and cancer (Olivieri *et al.* 2013). MiR-146a is encoded on human chromosome

5q33.3 and known to be essential modulator of differentiation and function of the cells of the innate as well as adaptive immunity (Rusca and Monticelli 2011, Roos *et al.* 2016). MiR-146a has been described as a negative regulator of signal pathways leading to nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation. Targets of miR-146a include tumor necrosis factor receptor-associated factor 6 (TRAF6) and interleukin-1 receptor-associated kinase 1 (IRAK1) genes, these act downstream of the Toll-like receptors (TLR) and interleukin-1 receptor (IL-1R) (Rusca and Monticelli 2011, Roos *et al.* 2016).

1.3 Menopause

Menopause is defined as the permanent cessation of a menstrual period that occurs naturally or as a result of surgery, radiation or chemotherapy. It is a natural physiological phenomenon in a woman's life as part of the normal aging process (Edwards and Li 2000). On average, woman becomes menopausal early in the sixth decade of life, with ethnic and regional variations (Baber *et al.* 2016). There are three menopause stages: pre-, peri- and postmenopause. Premenopause is the time before menopause when menstrual cycles are mainly regular. Perimenopause is the period when menopausal transition occurs and it continues until permanent cessation of menstrual cycle. In perimenopause menstrual cycle length is variable and women can experience two skipped cycles or amenorrhea at least 60 days. Postmenopause is time after permanent cessation of menstruation. It is defined retrospectively after 12 months of amenorrhea (Harlow *et al.* 2012).

There are additional symptoms than cessation of menses in climacteric. Other typical symptoms are vasomotor symptoms (hot flashes and night sweats), sleep disturbance and vaginal dryness. These symptoms can be treated with hormone replacement therapy (HRT) with estrogens often in combination with progestin (Edwards and Li 2000).

Hormonal changes during menopause are associated with significant increases in waist circumference and central abdominal fat. Aging and hormonal changes at menopause transition can both affect adipose tissue metabolism and thus the accumulation of body fat. Fat mass and visceral fat increases in non-obese women through menopausal transition. The redistribution of fat to the abdomen results in a transition from a gynoid to an android pattern of fat distribution (Davis *et al.* 2012, Baber *et al.* 2016). It is known that particularly visceral adipose tissue plays central role in the development of metabolic syndrome. Adipose tissue is an active endocrine organ that secretes cytokines, collectively called adipokines. Recent studies indicate that obesity causes chronic low-grade inflammation in adipose tissue. Also, a clear connection between low-grade inflammatory and metabolic diseases have described (Ouchi *et al.* 2011).

1.3.1 Estradiol

The steroid hormones are derived from cholesterol by side chain cleavage and oxidation and their synthesis also involve cytochrome P-450 enzymes. All steroid hormones act through nuclear receptors which act as transcription factors. The steroid hormones are synthesized by cells of the adrenal cortex and the gonads (e.g. estrogens). Estrogens are one class of steroid hormones that includes a group of chemically similar hormones: estrone (E_1), estradiol (E_2) and estriol (E_3). These hormones are important in female sexual and reproductive development. E_2, is synthesized from testosterone and its effects are mediated by the estrogen receptors ERα and ERβ. E_2 is the main circulating estrogen at fertile age but it decreases during menopause. As a result of decreased production of E_2 and loss of negative-feedback effect in the pituitary gland and the hypothalamus, the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) accelerates at the onset of menopause. After menopause main systemic estrogen is E_1 formed in adipose tissue and adrenal glands (Edwards and Li 2000, Nelson and Cox 2013, Yasar *et al.* 2016).

1.3.2 Follicle-stimulating hormone

Follicle-stimulating hormone (FSH) is a gonadotropin and belongs to glycoprotein hormone gene family. FSH is essential hormone of reproduction, development for gonadal, pubertal maturation and gamete production. FSH is secreted from anterior pituitary gland and use cyclic adenosine monophosphate (cAMP) as an intracellular messenger. FSH interact with cell surface receptors, located only in the gonads. The FSH receptor belongs to family of seven-transmembrane domain G-protein coupled receptor (Simony *et al.* 1997). A few years before menopause, menstruation becomes irregular and the number of ovarian follicles decreases. With ovarian follicles decreasing, ovarian production of E_2 ceases in the postmenopause phase. At the same time, production of FSH increases (Edwards and Li 2000).

2 AIM OF THE STUDY

This study was part of the Estrogenic Regulation of Muscle Apoptosis (ERMA) research project (Kovanen *et al.* 2018). The purpose of the ERMA research project is to understand the importance of E_2 deficiency associated with menopause, and to investigate molecular mechanisms of E_2 in the regulation of muscle size, composition, and performance of the middle-aged women.

The purpose of this Master's thesis was to determine the serum miR-21, miR-126 and miR-146a association with the risk factors of MetS, as well as association with menopause-related hormones FSH and E_2. The hypothesis was that circulating miR-21, miR-126 and miR-146a levels may vary in different MetS risk factor groups and stage of menopause.

Specific aims of the study:

1. Are serum miR-21, miR-126 and miR-146a levels associated with the metabolic syndrome?
2. Do the circulating miR-21, miR-126 and miR-146a levels associate with serum FSH and E_2 concentration in pre-, peri- and postmenopausal women?

3 MATERIALS AND METHODS

3.1 Participants and study design

Serum samples were collected from subjects who participated in the ERMA study (Kovanen *et al*. 2018). ERMA study was organized by The Gerontology Research Center (GEREC) at the Faculty of Sport and Health Sciences at the University of Jyväskylä. The participants were randomly selected from the age cohort of 47–55 years old women living in the area of Jyväskylä and categorized on the basis of their hormonal status. In order to participate in the study, subjects had to have at least one ovary. The exclusion criteria were: BMI > 35, external E_2 containing hormone preparation or other medications affecting ovarian function, currently being pregnant or lactating, polycystic ovary syndrome, chronic musculoskeletal disease, rheumatic disease, myocardial infarction, Crohn's disease, colitis ulcerosa, type 1 diabetes, type 2 diabetes with medication, diagnosed mental disorder, asthma with oral cortisol or cancer.

In this Master's thesis, a subgroup of ERMA study participants was selected (n =137) based on menopausal status and divided into pre-, peri- and postmenopausal groups. ERMA study design and subgroup of ERMA study participants selected in this Master's thesis is presented in (Figure 2).

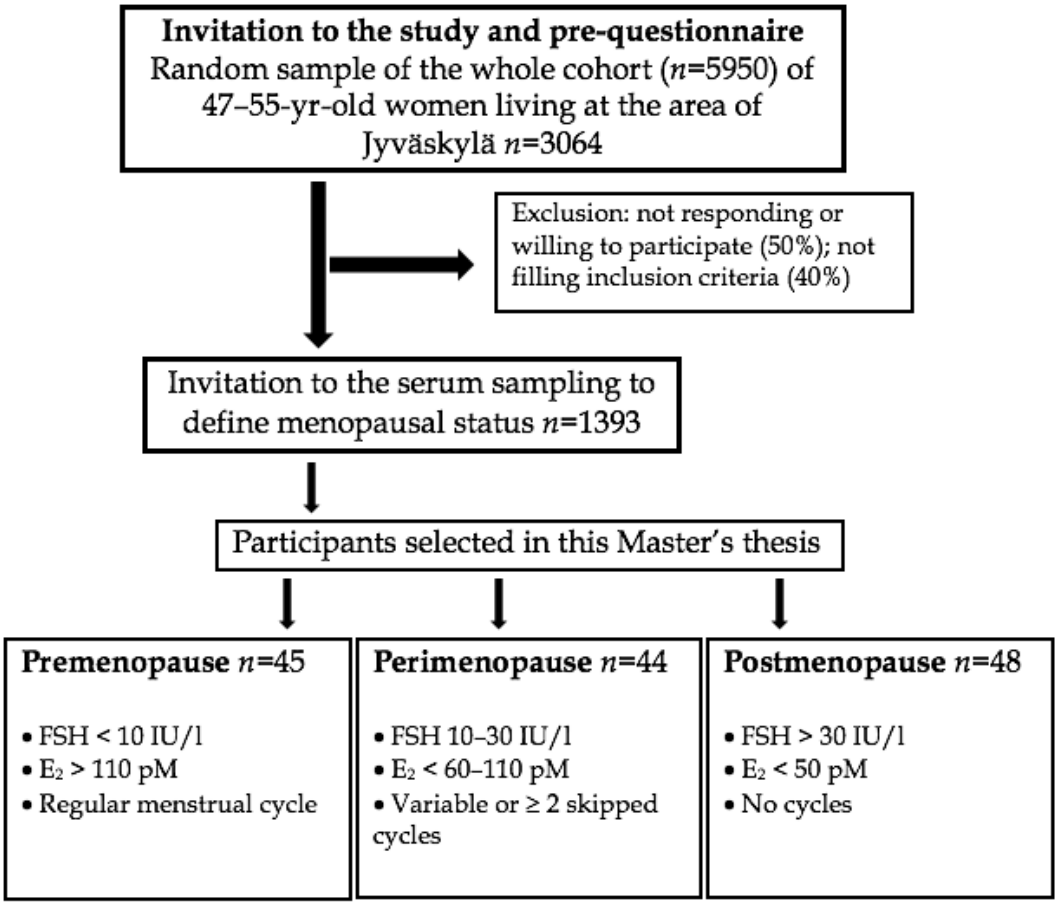

Figure 2. ERMA study design and subgroup of ERMA study participants (*n*=137) selected in this Master's thesis. Participants were divided on the basis of the menopausal status defined by blood test and menstrual diary.

Of the 1393 women, who were classified as pre-, peri- or postmenopausal, 137 were randomly selected to the current thesis based on the following criteria; stage of menopause and number of MetS risk factors they had. Following the IDF classification MetS groups were defined as; healthy (no risk factors), risk 1 (WC ≥ 80 cm), risk 2 (WC ≥ 80 cm and one additional risk factor; TG ≥ 1.7 mmol/l, HDL < 1.3 mmol/l, fasting glucose ≥ 5.6 mmol/l or SBP ≥ 130 mmHg and/or DBP ≥ 85 mmHg) and MetS (WC ≥ 80 cm and two or more additional risk factors). Participants having medical treatment for any of the MetS risk factors listed above were excluded from this study. Participants were distributed as evenly as possible both the risk factors for MetS and the menopausal groups (Table 2).

Table 2. Subjects were categorised by the risk factors of the metabolic syndrome and stage of menopause.

	PRE	PERI	POST	Total
HEALTHY	n=12	n=11	n=12	n=35
RISK 1	n=12	n=11	n=12	n=35
RISK 2	n=11	n=10	n=12	n=33
MetS	n=10	n=12	n=12	n=34
Total	n=45	n=44	n=48	n=137

The study protocol was approved by the Central Finland Health District Ethics Committee (Dnro 8U/2014) and was conducted according to the guidelines of the Declaration of Helsinki. All participants gave written informed consent before participating this study.

3.2 Laboratory assays

The participants fasted overnight and blood samples were drawn in a supine position. The serum samples were allowed to clot for 30 minutes at room temperature and serum was separated by centrifugation at 1900 x g for 15 minutes. Participants serum samples were analysed for fasting blood glucose and lipids (TC, HDL, TG) as well as hormones FSH and E_2. Hormones were analysed using Immulite® 2000xpi (Erlangen, Germany) analyser, based on two-site chemiluminescent immunometric assay (FSH) and enzyme-labelled chemiluminescent competitive immunoassay (E_2). Blood sugar and lipids were analysed spectrophotometrically using Konelab™ 20xt (Waltham, MA, USA) analyser.

3.3 Anthropometrics and blood pressure measurements

The height and body weight of the participants as well as their blood pressure and WC were measured at the beginning of the ERMA study. Height and weight were

used to calculate body mass index (BMI kg/m^2). Blood pressure was measured twice and averaged (OMRON M3 Intellisense™). WC measurements were repeated three times for a precise average measurement.

3.4 Total RNA isolation, cDNA synthesis and qPCR assay

The total RNA was extracted from 200 µl of serum using miRNeasy serum/plasma kit (QIAGEN, Valencia, CA, USA) according to manufacturer instruction. Synthetic *Caenorhabditis elegans* miR-39 spike-in control was used as an internal control and was added to samples after the addition of denaturant. Samples were eluted in 14 µl of RNase-free water. From each of the isolated RNA, 1 µl was collected and combined into one large pool for later use in qPCR as a reference sample. RNA concentrations were determined by NanoDrop ND-1000 (Wilmington, DE, USA) spectrophotometer.

RNA was reverse transcribed using miScript II RT Kit (QIAGEN) and miScript HiSpec buffer (for mature miRNA detection only). Mature miRNAs were polyadenylated by poly(A) polymerase and converted into cDNA with reverse transcriptase using oligo-dT primers, allowing amplification of mature miRNA in the real-time PCR step. The concentration of template RNA in reaction setup was around 100 ng. Total reaction volume was 20 µl and incubation was performed 60 min at 37 °C, followed by 5 min at 95 °C to inactivate miScript Reverse Transcriptase (Eppendorf, Mastercycler®).

In real-time PCR assays, miScript Primer Assay (QIAGEN) and miScript SYRB Green PCR kit (QIAGEN) was used as recommended by the manufacturer. miScript Primer Assay (forward primer) were specific for each miRNA: miR-39, miR-21, miR-126 and miR-146a. The miScript SYRB Green PCR kit contained miScript Universal primer (reverse primer) and QuantiTect SYBR Green PCR Master mix. Total

reaction volume was 25 µl per well (cDNA diluted 1:2) and 96-well plates were used. Cycling conditions for real-time PCR were 15 min at 95 °C to activate HotStarTag DNA polymerase followed by 40 cycles of 15 s denaturation at 94 °C, 30 s annealing at 55 °C and 30 s extension at 70 °C (C1000 Touch™ Thermal cycler, Bio-Rad). Each sample was tested in duplicate and melting curve analysis was used to verify the specific PCR-products.

3.5 Data normalization and statistical analysis

The data from qPCR assay was analysed using CFX manager™ software version 3.1 (Bio-Rad, Hercules, CA, USA). The threshold cycle (Ct) settings were automatic and threshold values and baseline were adjusted manually. Synthetic *C. elegans* miR-39 spike-in control was used to measure the RNA extraction efficiency. Added spike-in miRNA levels were measured in each serum analysis and was used for normalization of each sample. Values were calculated as ΔCt = mean Ct_{miRNA} – mean $Ct_{miRNA39}$. Relative quantities (RQ) were defined by using $RQ = 2^{-\Delta Ct}$ formula. The normalization factor (NF), the pool that was collected from each sample, was calculated for each sample $_j$ and was used to acquire the normalized relative quantities (NRQ) for each $miRNA_i$ and $sample_j$ as $NRQ_{ij} = RQ_{ij}/NF_j$ (Marabita *et al.* 2015).

Statistical analyses were performed using SPSS statistics software (IBM SPSS statistics 22, Chicago, IL, USA). All data were tested for normality by using Kolmogorov-Smirnov test (*K-S* test). One-way analysis of variance (ANOVA) test was used to compare significance between groups. MiRNA values were converted into a logarithmic form, repairing them normally distributed. Results were presented as the means ($\bar{x}$) and standard deviations (SD). A least significant difference (LSD) paired comparison test was used to analyse significance within groups. The significance level was a p-value < 0.05. Kruskal-Wallis test was used in

the case of non-normal distributions. Pearson correlation coefficient (*r*) was used to measure a statistical relationship between two variables.

4 RESULTS

4.1 Participants characteristics in a groups of metabolic risk factors

Participants age, anthropometrics, measured blood characteristics and serum miR-21, miR-126, miR-146a levels between groups according to the number of MetS groups are presented in (Table 3). Statistical significance in differences between studied groups (healthy, risk 1, risk 2 and MetS) are marked in the table.

There were no statistical differences in age, total cholesterol (TC), E_2, FSH, miR-21, miR-126 and miR-146a between any of the groups. However, a statistically significant difference was observed for BMI, WC, HDL, low density lipoprotein (LDL), TG, Gluc, SBP and DBP variables. The MetS group differed significantly from other groups in regard to WC, TG, HDL, Gluc, SBP and DBP values. From these values WC, TG, Gluc, SBP and DBP were highest in the MetS groups and lowest in the healthy group. The HDL values were lowest in the MetS group and highest in the healthy group. Also, comparison between healthy and risk 1 or risk 2 group followed same pattern, with higher values in risk 1 and risk 2 groups in WC, TG, Gluc, SBP and DBP variables. HDL values were lower in risk 1 and risk 2 groups compared to healthy group. However, in regard to Gluc and SBP the difference between the risk 1 group and healthy group was not significant.

Table 3. Age, anthropometrics and measured blood characteristics as well as serum miR-21, -126 and -146a levels between healthy, risk 1, risk 2 and MetS groups.

Variable	**Healthy** (*n*=35) $\bar{x}$ (SD)	**Risk 1** (*n*=35) $\bar{x}$ (SD)	**Risk 2** (*n*=33) $\bar{x}$ (SD)	**MetS** (*n*=34) $\bar{x}$ (SD)	ANOVA *p*-value
Age (years)	52.0 (2.1)	51.5 (2.0)	51.9 (1.7)	51.6 (1.9)	0.707
BMI (kg/m^2)	22.3 (1.8)	27.0 (2.5)[a]	28.0 (3.0)[a]	29.0 (3.3)[a, b]	<0.001
WC (cm)	73.1 (4.5)	87.1 (6.0)[a]	90.3 (7.8)[a]	94.0 (8.2)[a, b, c]	<0.001
TC (mmol/l)	5.1 (0.8)	5.5 (0.9)	5.5 (1.0)	5.3 (0.9)	0.225
HDL (mmol/l)	2.0 (0.4)	1.7 (0.3)[a]	1.7 (0.5)[a]	1.2 (0.4)[a, b, c]	<0.001
LDL (mmol/l)	2.9 (0.7)	3.4 (0.8)[a]	3.4 (0.8)[a]	3.5 (0.8)[a]	0.006
TG (mmol/l)	0.7 (0.2)	1.0 (0.3)[a]	1.2 (0.6)[a]	1.7 (0.8)[a, b, c]	<0.001[1]
Gluc (mmol/l)	4.8 (0.4)	4.9 (0.4)	5.2 (0.5)[a, b]	5.4 (0.6)[a, b, c]	<0.001
SBP (mmHg)	121.0 (11.1)	124.3 (8.6)	131.5 (13.1) [a, b]	143.8 (14.0) [a, b, c]	<0.001
DBP (mmHg)	75.9 (5.7)	80.0 (4.3)[a]	85.2 (8.7) [a, b]	93.1 (7.8) [a, b, c]	<0.001
E_2 (pmol/l)	410.1 (446.0)	314.3 (279.0)	289.6[2] (218.5)	212.6 (131.1)	0.052[1]
FSH (IU/l)	47.3 (43.8)	46.8 (45.1)	40.6 (32.6)	38.0 (30.5)	0.667
miR-21	0.6 (0.5)	0.5 (0.6)	0.4 (0.3)	0.4 (0.3)	0.779[1]
miR-126	0.7 (0.5)	0.7 (0.6)	0.6 (0.7)	0.6 (0.4)	0.361[1]
miR-146a	0.6 (0.5)	0.6 (0.5)	0.6 (0.5)	0.5 (0.3)	0.969[1]

[1] Non-parametric Kruskal Wallis test [2] one marked as an outlier and was excluded. Values are the means ($\bar{x}$) and standard deviation (SD). After statistically significant ANOVA p-value, LSD paired comparison test was used to locate which of the groups differ from each other; [a] $p<0.05$ compared to healthy group, [b] $p<0.05$ compared to risk 1 group and [c] $p<0.05$ compared to risk 2 group.
Abbreviations: BMI, body mass index; WC, waist circumference; TC, total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; TG, triglycerides; Gluc, glucose; SBP, systolic blood pressure; DBP, diastolic blood pressure; E_2, estradiol; FSH, follicle-stimulating hormone.

4.2 Participants characteristics in a groups of menopausal status

Table 4 shows the participants age, anthropometrics, measured blood characteristics and serum miR-21, miR-126, miR-146a levels between pre-, peri- and postmenopause groups. Statistical significance in differences between studied groups (pre-, peri- and postmenopause) are marked in the table.

There were no statistical differences in BMI, WC, HDL, TG, Gluc, SBP, DBP, miR-21, miR-126 and miR-146a between pre-, peri- or postmenopausal groups. Statistical difference was observed for age, hormones FSH and E_2, TC and LDL variables. Serum E_2 levels were higher in the premenopause group and serum FSH levels in the postmenopause group, respectively. TC values were significantly higher in postmenopause group compared to the pre- and perimenopause group. Also, LDL values were higher in postmenopause group compared to the perimenopause group.

Table 4. Age, anthropometrics and measured blood characteristics as well as serum miR-21, -126 and -146a levels between pre-, peri- and postmenopause groups.

Variable	**Premeno-pause** (*n*=45) $\bar{x}$ (SD)	**Perimeno-pause** (*n*=44) $\bar{x}$ (SD)	**Postmeno-pause** (*n*=48) $\bar{x}$ (SD)	ANOVA *p*-value
Age (years)	51.0 (1.6)	51.9 (1.9)[a]	52.3 (2.1)[a]	0.004
BMI (kg/m²)	26.2 (3.5)	27.3 (4.0)	26.1 (3.6)	0.241
WC (cm)	85.0 (9.3)	87.4 (11.7)	86.0 (10.1)	0.429
TC (mmol/l)	5.2 (0.9)	5.1 (0.8)	5.7 (0.9)[a, b]	0.002
HDL (mmol/l)	1.6 (0.4)	1.6 (0.5)	1.7 (0.6)	0.652
LDL (mmol/l)	3.2 (0.9)	3.1 (0.8)	3.5 (0.7)[b]	0.039
TG (mmol/l)	1.1 (0.5)	1.1 (0.6)	1.3 (0.7)	0.303[1]
Gluc (mmol/l)	5.2 (0.5)	5.1 (0.5)	5.0 (0.5)	0.161
SBP (mmHg)	129.4 (15.6)	130.0 (12.2)	130.7 (16.0)	0.289
DBP (mmHg)	81.5 (8.1)	83.8 (8.2)	84.5 (11.2)	0.912
E_2 (pmol/l)	500.7[2] (414.2)	290.0 (197.1)[a]	147.0 (62.9)[a, b]	<0.001[1]
FSH (IU/l)	9.3 (3.8)	34.5 (24.3)[a]	82.8 (31.0)[a, b]	<0.001
miR-21	0.4 (0.3)	0.5 (0.5)	0.5 (0.6)	0.725[1]
miR-126	0.6 (0.5)	0.8 (0.6)	0.7 (0.5)	0.372[1]
miR-146a	0.5 (0.3)	0.6 (0.4)	0.6 (0.5)	0.348[1]

[1] Non-parametric Kruskal Wallis test [2] one marked as an outlier and was excluded. Values are the means ($\bar{x}$) and standard deviation (SD). After statistically significant ANOVA *p*-value, LSD paired comparison test was used to locate which of the groups differ from each other; [a] $p<0.05$ compared to premenopause group, [b] $p<0.05$ compared to perimenopause group.

Abbreviations: BMI, body mass index; WC, waist circumference; TC, total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; TG, triglycerides; Gluc, glucose; SBP, systolic blood pressure; DBP, diastolic blood pressure; E_2, estradiol; FSH, follicle-stimulating hormone.

4.3 Correlation between studied variables

Table 5 presents the correlation analysis of the participant's anthropometrics, measured blood characteristics and serum miR-21, miR-126, miR-146a levels. Circulating miRNAs had a strong positive correlation with each other. Serum miR-21 and miR-126 had negative correlation with glucose and miR-21 and E_2 had negative correlation with each other. Strong positive correlation was observed between BMI and WC. Also, there was a statistically significant correlation between HDL, LDL, TG, Gluc, SBP and DBP. All lipids correlated with each other. FSH and E_2 had a negative correlation with each other. Additionally, FSH correlated with cholesterols and E_2 correlated with WC, TC and LDL.

Table 5. Pearson correlation of participant's anthropometrics and measured blood characteristics as well as serum miR-21, -126 and -146a levels between two variables.

	BMI	WC	TC	HDL	LDL	TG	GLUC	SBP	DBP	E_2	FSH	miR-21	miR-126
WC	**.87****												
TC	.08	.11											
HDL	**-.36****	**-.47****	**.18***										
LDL	**.22***	**.29****	**.86****	**-.25****									
TG	**.40****	**.55****	**.37****	**-.54****	**-.50****								
GLUC	**.38****	**.34****	.15	**-.21***	**.20***	**.30****							
SBP	**.33****	**.39****	.03	**-.19***	.08	**.33****	**.26****						
DBP	**.47****	**.54****	.03	**.29****	.09	**.40****	**.24****	**.81****					
E_2	-.15	**-.17***	**-.22***	.08	**-.23****	-.13	-.07	-.11	-.17				
FSH	-.04	-.02	**.31****	**.17***	**.20***	-.02	-.13	-.02	.01	**-.57****			
miR-21	-.07	-.11	-.05	.13	-.06	-.11	**-20***	.09	.02	**-.22***	.12		
miR-126	-.04	-.05	-.01	.15	-.06	-.07	**-.24****	.05	.02	-.16	.08	**.77****	
miR-146a	-.04	-.03	-.01	.12	-.03	-.01	-.10	.16	.03	-.14	.10	**.80****	**.69****

Statistically significant results are marked as bold.
** Correlation is significant at the < 0.01 level. * Correlation is significant at the < 0.05 level.
Abbreviations: BMI, body mass index; WC, waist circumference; TC, total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; TG, triglycerides; Gluc, glucose; SBP, systolic blood pressure; DBP, diastolic blood pressure; E_2, estradiol; FSH, follicle-stimulating hormone.

5 DISCUSSION

Today obesity and overweight have reached epidemic proportions. These conditions may cause different kinds of complications such as MetS (Alberti *et al.* 2009). Nowadays miRNAs are under extensive research, since they have shown to be potential biomarkers for different conditions. Therefore, these tiny regulatory RNAs may be use for risk estimation and classification in the future (Iacomino and Siani 2017). The purpose of this study was to determine the association between serum miR-21, miR-126 and miR-146a to the risk factors of metabolic syndrome. Secondly, the association of these above-mentioned miRNAs with menopause-related hormones FSH and E_2 and menopausal status. This study could not establish a distinct association between serum miR-21, miR-126 and miR-146a to the risk factor groups of metabolic syndrome or the serum FSH and E_2 concentration in middle-aged women. However, significant correlation was observed between glucose and miR-21 and miR-126. Also, significant correlation between E_2 and miR-21 was observed.

5.1 Association between serum miR-21, miR-126 and miR-146a to the risk factors of metabolic syndrome

Results indicate that there was no association between serum miR-21, miR-126, miR-146a and the risk factors of MetS. Results show that statistical significance was in BMI, WC, HDL, LDL, TG, Gluc, SBP and DBP variables in the risk factor of the metabolic syndrome groups. All of these statistically significant variables: WC, HDL, TG, Gluc, SBP and DBP are risk factors of MetS, thereby these results are as anticipated (Alberti *et al.* 2009). In addition, a statistically significant difference was also observed between BMI and LDL variables. BMI and WC are related to each other, but they measure different aspects of obesity. WC is a suitable marker of

visceral fat mass and BMI is well reflected in the total amount of body fat tissue and is, therefore, a good weight indicator. On the other hand, BMI does not specify fat distribution in the body or take into consideration the composition of the body. Therefore, WC is a more suitable indicator for MetS, but both WC and BMI are relevant predictors of obesity-related health risks. Even though LDL is not one of the MetS risk factors, it is considered a major risk factor for CVD (Bairaktari *et al.* 2005). Both LDL and HDL play an important role in cholesterol transfer and metabolism. LDL particles carry cholesterol to the tissues, including the arteries walls causing atherosclerosis. HDL particles have a role in reverse cholesterol transport from the tissues to the liver, respectively (Daniels *et al.* 2009).

5.2 Association between circulating miR-21, miR-126 and miR-146a and stage of menopause

These results suggest that there was no association between circulating miR-21, miR-126 and miR-146a with the menopause stage. As expected, there was a statistical difference in age, hormones FSH and E_2, TC and LDL variables according to the menopause stage of the subjects. Postmenopausal women were a bit older than pre- and perimenopausal women. FSH-levels increased and E_2-levels decreased during the menopausal transition in a manner described in the literature (Edwards and Li 2000). We observed a statistically significant difference in TC and LDL between the menopause groups, which is in line with a previous study showing that TC and LDL increases due to menopause-induced changes (Matthews *et. al* 2009). The mean levels of TC were significantly higher in postmenopausal women than in pre- and perimenopausal women, while LDL were significantly higher in postmenopausal women than in perimenopausal women. It is known that loss of circulating E_2 is associated with threatening lipid profile (Edwards and Li 2000). Also, the increase in CVD and MetS in postmenopausal women may partly be related to the changes in lipids associated with the menopausal transition (Carr

2003). All of the variables showed statistical difference can be explained due to an association of menopausal transition.

5.3 Circulating miRNAs correlated with glucose and hormone E_2

In this study there was a statistically significant correlation of miR-21 and miR-126 with two biochemical variables. Significant correlation was observed between glucose and miR-21 and miR-126. In addition, significant correlation between E2 and miR-21 was observed. Also, selected circulating miRNAs had a positive correlation with each other. This may indicate that selected miRNAs are either directly or indirectly regulated by the hormone level or blood glucose concentration. There are numerous studies shown selected miR-21, miR-126 and/or miR-146a to be associated for example T2DM and CVD (Zampetaki *et al.* 2010, Zampetaki and Mayer 2012, Olivieri *et al.* 2015, Nunez Lopez *et al.* 2016). Also, E_2 and miR-21 association has been demonstrated (Klinge 2009, Wickramasinghe *et al.* 2009). It is known that obesity alters miRNA expression in metabolically important organs. Also, miRNAs regulate multiple pathways such as lipid metabolism and insulin signaling (Deiuliis 2016). Tao *et al.* (2016) have shown that miR-126 inhibits the β-cell proliferation through its target insulin receptor substrate 2 (IRS-2) instead of insulin receptor substrate 1 (IRS-1), and may take part in the glucose homeostasis. Also, miR-21 is linked to insulin-resistance deterioration within progression from obesity to T2DM (Guglielmi *et al.* 2017).

5.4 Strengths and limitations

There are some limitations in this study. The overall amount of miRNAs presents in the serum samples is quite low. In addition, lack of endogenous controls and standardized methods causes own challenges for the study. On the other hand, this study has its strengths, number of randomly-selected participants and use of spike-in control. Even though, there are challenges in miRNA experiments, the same

protocols were used throughout the study. Therefore, the possible bias would be similar in each sample and would not affect the results abnormally.

5.5 Future prospects

Circulating miRNAs are easily detectable in blood or other body fluids. Therefore, it is an ideal candidate for a biomarker. However, preanalytical and analytical variables affecting the quantification of miRNA in serum samples (McDonald *et al.* 2011). There are many challenges in miRNA experiments regarding reproducibility, lack of standardized protocols and different quantification methods (Iacomino and Siani 2017). Also, it is crucial to select a suitable RNA isolation method and amount of input material (El-Khoury *et al.* 2016). In addition, origin and function of circulating miRNAs are still partially remained unclear (Polakovicova *et al.* 2016, Iacomino and Siani 2017). In the future, more detailed knowledge of the miRNAs function and action is needed before miRNAs can be established as clinically useful biomarkers.

5.6 Conclusions

This study aimed to determine the serum miR-21, miR-126 and miR-146a association with the risk factors of metabolic syndrome, as well as association with menopause-related hormones FSH and E_2. The hypothesis was that circulating miR-21, miR-126 and miR-146a levels may vary in different groups according to the risk factors of the metabolic syndrome and stage of menopause. This association could not be shown in this study. Even though this study did not find an association between selected miRNAs and the risk factors of metabolic syndrome, statistically significant correlation was found between glucose and miR-21, glucose and miR-126, miR-21 and E_2. In summary, based on the results of this thesis, despite the associations found between miRNAs and glucose, the studied miRNAs cannot be considered as markers of metabolic syndrome.

REFERENCES

Alberti K.G.M.M, Eckel R.H., Grundy S.M. Zimmet P.Z., Cleeman J.I., Donato K.A., Fruchart J.C., James W.P., Loria C.M. and Smith S.C. 2009. Harmonizing the Metabolic Syndrome: A Joint Interim Statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation* 120: 1640–1645.

Baber R.J., Panay N., Fenton A. & IMS Writing Group. 2016. 2016 IMS Recommendations on women's midlife health and menopause hormone therapy. *Climacteric* 19: 109–150.

Bairaktari E.T., Seferiadis K.I. & Elisaf M.S. 2005. Evaluation of methods for the measurement of low-density lipoprotein cholesterol. *J Cardiovasc Pharmacol Ther* 10: 45–54.

Carr M.C. 2003. The emergence of the metabolic syndrome with menopause. *J Clin Endocrinol Metab* 88: 2404–2411.

Chen X., Ba Y., Ma L., Cai X., Yin Y., Wang K., Guo J., Zhang Y., Chen J., Guo X., Li Q., Li X., Wang W., Zhang Y., Wang J., Jiang X., Xiang Y., Xu C., Zheng P., Zhang J., Li R., Zhang H., Shang X., Gong T., Ning G., Wang J., Zen K., Zhang J. & Zhang C-Y. 2008. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res* 18: 997–1006.

Daniels T.F., Killinger K.M., Michal J.J., Wright Jr. R.W. & Jiang Z. 2009. Lipoproteins, cholesterol homeostasis and cardiac health. *Int J Biol Sci* 5: 474–488.

Davis S.R., Castelo-Branco C., Chedraui P., Lumsden M.A., Nappi R.E., Shah D. & Villaseca P; Writing Group of the International Menopause Society for World Menopause Day 2012. 2012. Understanding weight gain at menopause. *Climacteric* 15: 419–429.

Deiuliis J.A. 2016. MicroRNAs as regulators of metabolic disease: pathophysiologic significance and emerging role as biomarkers and therapeutics. *Int J Obes* 40: 88–101.

Eckel R.H., Grundy S.M. & Zimmet P.Z. 2005. The metabolic syndrome. *Lancet* 365: 1415–1428.

Edwards B.J. & Li J. 2000. Endocrinology of menopause. *Periodontology* 61: 177–194.

El-Khoury V., Pierson S., Kaoma T., Bernardin F. & Berchem G. 2016. Assessing cellular and circulating miRNA recovery: the impact of the RNA isolation method and the quantity of input material. *Sci Rep.* 6: 19529.

Guglielmi V., D'Adamo M., Menghini R., Cardellini M., Gentileschi P., Federici M. & Sbraccia P. 2017. MicroRNA 21 is up-regulated in adipose tissue of obese diabetic subjects. *Nutr Healthy Aging* 4: 141–145.

Ha M. & Kim V.N. 2014. Regulation of microRNA biogenesis. *Nat Rev Mol Cell Biol* 15: 509–524.

Harlow S.D., Gass M., Hall J.E., Lobo R., Maki P., Rebar R.W., Sherman S., Sluss P.M., de Villiers T.J. & for the STRAW + 10 Collaborative Group. 2012. Executive summary of the Stages of Reproductive Aging Workshop + 10: addressing the unfinished agenda of staging reproductive aging. *J Clin Endocrinol Metab* 97: 1159–1168.

Iacomino G. & Siani A. 2017. Role of microRNAs in obesity and obesity-related diseases. *Genes Nutr.* 25: 12–23.

International diabetes federation (IDF). 2015. IDF worldwide definition of the metabolic syndrome. http://www.idf.org/metabolic-syndrome. Retrieved on 24.2.2017.

Kangas R., Pöllänen E., Rippo M.R., Lanzarini C., Prattichizzo F., Niskala P., Jylhävä J., Sipilä S., Kaprio J., Procopio A.D., Capri M., Franceschi C., Olivieri F. & Kovanen V. 2014. Circulating miR-21, miR-146a and Fas ligand respond to postmenopausal estrogen-based hormone replacement therapy – A study with monozygotic twin pairs. *Mech Ageing Dev* 15: 143–144.

Kangas R., Törmäkangas T., Fey V., Pursiheimo J., Miinalainen I., Alen M., Kaprio J., Sipilä S., Säämänen A-M., Kovanen V. & Laakkonen E.K. 2017. Aging and serum exomiR content in women-effects of estrogenic hormone replacement therapy. *Sci Rep* 7: 42702

Kim V.N., Han J. & Siomi M.C. 2009. Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol* 10: 126–139.

Klinge C.M. 2009. Estrogen Regulation of MicroRNA Expression. *Curr Genomics* 10: 169–183.

Kovanen V., Aukee P., Kokko K., Finni T., Tarkka I.M., Tammelin T., Kujala U.M., Sipilä S. & Laakkonen E.K. 2018. Design and protocol of Estrogenic Regulation of Muscle Apoptosis (ERMA) study with 47 to 55-year-old women's cohort: novel results show menopause-related differences in blood count. *Menopause* 25: 1020–1032.

Lagos-Quintana M., Rauhut R., Lendeckel W. & Tuschi T. 2001. Identification of novel genes coding for small expressed RNAs. *Science* 294: 853–858.

Lau N.C., Lim L.P., Weinstein E.G. & Bartel D.P. 2001. An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science* 294: 858–862.

Lee R.C., Feinbaum R.L. & Ambros V. 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 75: 843–854.

Lee R.C. & Ambros V. 2001. An extensive class of small RNAs in Caenorhabditis elegans. *Science* 294: 862–864.

Lopez-Ramirez M.A, Reijerkerk A., de Vries H.E. & Romero I.A. 2016. Regulation of brain endothelial barrier function by microRNAs in health and neuroinflammation. *FASEB J.* 30: 2662–72.

MacFarlane LA. & Murphy P.R. 2010. MicroRNA: Biogenesis, Function and Role in Cancer. *Curr Genomics* 11: 537–561.

Marabita F., de Candia P., Torri A., Tegner J., Abrignani S. & Rossi R.L. 2015. Normalization of circulating microRNA expression data obtained by quantitative real-time RT-PCR. *Briefings in Bioinformatics* 10: 1–9.

Matthews K.A., Crawford S.L., Chae C.U., Everson-Rose S.A., Sowers M.F., Sternfeld B. & Sutton-Tyrrel K. 2009. Are changes in cardiovascular disease risk factors in midlife women due to chronological aging or to the menopausal transition? *J Am Coll Cardiol* 15: 2366–73.

McDonald J.S., Milosevic D., Reddi H.V., Grebe S.K. & Algeciras-Schimnich A. 2011. Analysis of circulating microRNA: preanalytical and analytical challenges. *Clin Chem* 57: 833–40.

Mitchell P.S., Parkin R.K., Kroh E.M., Fritz B.R., Wyman S.K., Pogosova-Agadjanyan E.L., Peterson A., Noteboom J., O'Briant K.C., Allen A., Lin D.W., Urban N., Drescher C.W., Knudsen B.S., Stirewalt D.L., Gentleman R., Vessella R.L., Nelson P.S., Martin D.B. & Tewari M. 2008. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Asad Sci U S A* 105: 10513–8.

Musilova K. & Mraz M. 2015. MicroRNAs in B-cell lymphomas: how a complex biology gets more complex. *Leukemia* 29: 1004–17

Nelson D.L. & Cox M.M. 2013. *Lehninger Principles of Biochemistry*. W.H Freeman Co Ltd. 6 th edition. 1158 pp.

Nunez Lopez Y.O., Garufi G. & Seyhan A.A. 2016. Altered Levels of Circulating Cytokines and microRNAs in Lean and Obese Individuals with Prediabetes and Type 2 Diabetes. *Mol Biosyst* 13: 106–121.

Olivieri F., Rippo M.R., Procopio A.D. & Fazioli F. 2013. Circulating inflamma-miRs in aging and age-related diseases. *Front Genet* 4: 121.

Olivieri F., Spazzafumo L., Bonafe M., Recchioni R., Prattichizzo F., Marcheselli F., Micolucci L., Mensa E., Giuliani A., Santini G., Gobbi M., Lazzarini R., Boemi M., Testa R., Antonicelli R., Procopio A.D. & Bonfigli A.R. 2015. MiR-21-5p and miR-126a-3p levels in plasma and circulating angiogenic cells: relationship with type 2 diabetes complications. *Oncotarget* 6: 35372–82.

Ouchi N., Parker J.L., Lugus J.J. & Walsh K. 2011. Adipokines in inflammation and metabolic disease. *Nat Rev Immunol* 11: 85–97.

Polakovicova M., Musil P., Laczo E., Hamar D. & Kyselovic J. 2016. Circulating MicroRNAs as Potential Biomarkers of Exercise Response. *Int J Mol Sci* 17: 1553.

Reid G., Kirschner M.B. & van Zandwijk N. 2011. Circulating microRNAs: Association with disease and potential use as biomarkers. *Crit Rev Oncol Hematol* 80: 193–208.

Roos J., Enlund E., Funcke J-B., Tews D., Holzmann K., Debatin K-M., Wabitsch M. & Fischer-Posovszky P. 2016. miR-146a-mediated suppression of the inflammatory response in human adipocytes. *Sci Rep* 6: 38339.

Rusca N. & Monticelli S. 2011. MiR-146a in immunity and Disease. *Mol Biol Int* 2011: 437301.

Simoni M., Gromoll J. & Nieschlag E. 1997. The Follicle-Stimulating Hormone Receptor: Biochemistry, Molecular Biology, Physiology, and Pathophysiology. *Endocr Rev* 18: 739–773.

Sekar D., Saravanan S., Karikalan K., Thirugnanasambantham K., Lalitha P. & Islam V.I.H. 2015. Role of MicroRNA 21 in Mesenchymal Stem Cell (MSC) Differentiation: A Powerful Biomarker in MSCs Derived Cells. *Curr Pharm Biotechnol* 16: 43–48.

Tao H., Wang M-m., Zhang M., Zhang S-p., Wang C-h., Yuan W-j., Sun T., He L-j. & Hu Q-k. 2016. MiR-126 Suppresses the Glucose-Stimulated Proliferation via IRS-2 in INS-1 β Cells. *PLoS One* 11: e0149954.

Turchinovich A., Weiz L., Langheinz A. & Burwinkel B. 2011. Characterization of extracellular circulating microRNA. *Nucleic Acids Res* 39: 7223–7233.

Villard A., Marchand L., Thivolet C. & Rome S. 2015. Diagnostic Value of Cell-free Circulating MicroRNAs for Obesity and Type 2 Diabetes: A Meta-analysis. *J Mol Biomark Diagn* 6: 251.

Wickramasinghe N.S., Manavalan T.T., Dougherty S.M., Riggs K.A., Li Y. & Klinge C.M. 2009. Estradiol downregulates miR-21 expression and increases miR-21 target gene expression in MCF-7 breast cancer cells. *Nucleic Acids Res* 37: 2584–2595.

Yasar P., Ayaz G., User S.D., Güpür G. & Muyan M. 2016. Molecular mechanism of estrogen-estrogen receptor signaling. *Reprod Med Biol* 16: 4–20.

Zampetaki A., Kiechl S., Drozdov I., Willeit P., Mayr U., Prokopi M., Mayr A., Weger S., Oberhollenzer F., Bonora E., Shah A., Willeit J. & Mayer M. 2010. Plasma MicroRNA Profiling Reveals Loss of Endothelial MiR-126 and Other MicroRNAs in Type 2 Diabetes. *Circ Res* 107: 810–7.

Zampetaki A. & Mayer M. 2012. MicroRNAs in vascular and metabolic disease. *Circ Res* 110: 508–22.